ALTERATION IN OLIGODENDROGLIAL LINEAGE PROGRESSION FOLLOWING EXPOSURE TO BPA IN VITRO

Honors Fellow Thesis

by

GRACE PHILIP

Submitted to Honors and Undergraduate Research
Texas A&M University
in fulfillment of the requirements for the designation as

HONORS UNDERGRADATE RESEARCH FELLOW

May 2012

Major: Biomedical Science

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Approved by:

Research Advisor: C. Jane Welsh
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ABSTRACT

Alteration in Oligodendroglial Lineage Progression Following Exposure to BPA *in Vitro*. (May 2012)

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Multiple Sclerosis is an autoimmune disease characterized by demyelination and remyelination resulting in motor, sensory, visual, and autonomic dysfunctions that accumulate over time. Demyelination is thought to be caused by autoreactive lymphocytes that cross the highly regulated blood-brain barrier made of cerebral endothelial cells. Oligodendrocytes in the central nervous system synthesize myelin-related proteins, but a malfunction in the cell differentiation may contribute to improper myelination of the neuron or the inability to remyelinate after an autoimmune attack. A key environmental risk factor for MS is thought to be the widely used plasticizer bisphenol A (BPA). This study investigated the role on BPA as an etiologic agent of MS progression by investigating the changes induced in the OPC maturation cycle through BPA exposure at different concentrations. Quantification through differential immunohistochemical staining was analyzed by statistical tests.

DEDICATION

This thesis and, in fact, my entire education is dedicated to my family who have brought me this far and supported me for this long. I am so privileged that my family is still growing with new additions this year. I would be remiss without mentioning my dad George Philip, mom Marykutty Philip, brother Steven Philip, sister Joyce Philip, future brother-in-law Praise Rajan, and future sister-in-law Lydia Daniel.

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CHAPTER I

INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune inflammatory disease of the brain and spinal cord. The early disease is characterized by demyelination and remyelination that is clinically manifested in neurological dysfunctions of the motor, sensory, visual, and autonomic systems. This demyelinating disease is pathognomonically characterized by the formation of sclerotic plaques which are areas of inflammation and demyelinated axons. Clinically, 80% of patients with the above listed manifestations of demyelination undergo a relapsing-remitting cycle of multiple sclerosis where the patients have an incomplete recovery after each episode only to experience persistent, disabling, and accumulating symptoms in the future. Eventually at around 40 years of age, or after several decades the disease progresses and evolves to cause complications due to infections of the skin, chest, and bladder, depression from cerebral inflammation or the restrictions and uncertainties in nearly half of the patient sample, or death in nearly two-thirds of the cases (Compston and Coles, 2008).

Axons are only able to propagate the electrochemical signal in neurons effectively through insulation provided by myelin. Myelin is a protein-lipid complex that wraps

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This thesis follows the style of Journal of Neuroimmunology

around the axon and is secreted by oligodendrocytes, a macroglial cell present in the Central Nervous System (CNS). Oligodendrocytes exist first as pleuripotent neural stem cells, second as oligodendrocytes progenitor cells (OPC) in the dorsal spinal cord and telecephalon, then finally as pre-oligodendrocytes. Upon stimulation, OPC migrate to developing axons, exit the cell cycle, and differentiate into mature oligodendrocytes that produce myelin- related proteins. The oligodendrocytes continue to maintain the myelin membrane which consists of continual turnover of myelin and high myelin gene expression (Miron et al., 2010). Demyelination occurs as lymphocytes cross the blood-brain barrier (BBB) and attack the myelin covering. The BBB is an organization of cerebral endothelial cells that separate the cerebral vascular compartment from the cerebral interstitium. A functioning BBB sequesters the CNS from influx of blood elements and humoral neurotransmitters; consequently, breakdown or dysregulation of this barrier allows migration of activated leukocytes (Minagar and Alexander, 2003). Therefore, in demyelination, the potassium ions leakage results in a failure to conduct action potentials and is clinically manifested in motor and sensory dysfunctions (Compston and Coles, 2008; Standring and Gray, 2008)

There are several factors that can lead to or exacerbate the condition of Multiple Sclerosis in a population. Environmentally, there is a generalized increase in global distribution at latitudes at a greater distance from the equator. For example, the disease is common in the Northern European population. Multiple Sclerosis has also been associated with infections of measles, mumps, rubella, and Epstein- Barr virus at later

ages and also a higher risk of relapse after upper respiratory and gastrointestinal infections. Other environmental triggers can be due to low sunlight, vitamin D deficiency, smoking, and pollutants (Compston and Coles, 2008). Multiple Sclerosis is also more common in females than males with the trend of decreasing relapse rates during pregnancies and increases sensitivity to estrogen levels (Whitacre et al., 1999).

In the last half century there has been an increased incidence of Multiple Sclerosis worldwide. Although this increase may be attributed to the improved methods of detection and reporting, researchers hypothesize the change may arise from environmental risk factors (Noonan et al., 2001). The most noteworthy environmental risk factor includes the abundant use of bisphenol A (BPA). BPA is a plasticizer used worldwide in the production of plastics and epoxy resins in most everyday products. Its use includes food and drink containers, dental enamels, and linings of food cans. BPA has been found in body fluids, fetal serum, and full-term amniotic fluid. It can enter the body by product contamination, leakage from these food containers into the food, or contact with products (Vandenberg et al., 2007; Whitacre et al., 1999). Its presence in these body fluids indicates that BPA can pass the placental barrier, though it is found at higher levels in the maternal blood because of the hemochorial placentation of humans (Brown Jr., 2009). However, given the passing of BPA to the fetus, there is a high chance that the developing fetus will contain this compound at high levels during the formative weeks of neurological development.

Bisphenol A is classified into a group of drugs that are known as endocrine disrupting compounds (EDC). EDC are a class of environmental contaminants that cause an abnormal endocrine milieu in the human body. BPA, in particular, imitates estrogen and binds to estrogen receptors (Welshons et al., 2003). Once bound, BPA sets off a cascade of endocrine related diseases that are naturally supposed to have activation from the endogenous source of estrogen and not exogenous estrogenic imitators (Yamaguchi et al., 2006). For this reason, BPA is implicated for schizophrenia, prostate cancer, early puberty, neoplastic lesions, and reduced mammary gland and altered breast development (Diamanti-Kandarakis et al., 2009).

In addition to its presence, BPA was discovered to have neurological effects in astrocyte progenitor cells. Astrocytes are found in abundance exclusively in the central nervous system and have a variety of functions including forming the highly selective blood brain barrier, functioning as antigen presenting cells to T-cells, and recruiting macrophages in the central nervous system with chemokines (Minagar et al., 2002). At *in vitro* dosages, BPA causes serum-free mouse embryo cells, the astrocyte progenitor cell, to increase production of glial fibrillary acidic protein, thereby showing that BPA causes astrocytes to mature fast. This poses a problem *in vivo* because astrocytes normally appear in the brain during the late terms and early postnatal stages to help guide axons to the desired and appropriate synapse (Yamaguchi et al., 2006).

BPA is found to bind to thyroid hormone receptors and antagonize the production of T3 thereby reducing the production of T3 gene expression *in vitro* (Zoeller 2005). This finding is significant because the oligodendrocytes differentiate and mature in the active form through binding of thyroid hormone to the thyroid hormone receptor. Therefore, the lack of thyroid hormone presence in the developing brain will inhibit the myelination of axons in the fetus (Seiwa et al., 2004). The negative effects of the thyroid hormone inhibition due to BPA can be traced to the myelin basic protein (MBP). MBP is found to be upregulated in the brain due to thyroid hormone release. MBP plays a key role as a protein in the overall structure of myelin, the phospholipid membrane that protects and insulates the axon and travelling electrochemical signal of the neuron (Farsetti et al., 1991). However in a brain undergoing secondary hypothyroidism due to BPA, there is a marked decrease in MBP production (Zoeller et al., 2000). This finding has been substantiated in an *in vitro* study that found BPA to inhibit the expression of MBP at doses of 10^{-5} M (Seiwa et al., 2004). Due to all of the research supporting the physiological and endocrine disrupting effects of BPA, the current investigation focuses on the potential role of BPA in the etiology of MS by examining its effects on oligodendroglial cells.

Objectives

The purpose of this project was to investigate the effect of BPA on oligodendrocytes development in order to explore the relationship between Multiple Sclerosis and BPA. Previous studies have focused on specific aspects of the disease, but this approach for assessing the developmental changes of oligodendrocytes and provides holistic information about the role on BPA on OPC maturation. Furthermore, this project provides more information about the myelination process of oligodendrocytes and susceptibility to Multiple Sclerosis by assessing the cytotoxic, maturation, and proliferative effects of BPA on oligodendrocytes progenitor cells.

CHAPTER II

METHODS

Primary mixed glial cultures were prepared from the forebrains of 1- to 2-day-old C57Bl/6 mice using a differential detachment method as detailed previously (Li et al. 2008) and provided to Dr. Brinkmeyer and Ms Philip for analysis. Briefly, mixed glial cultures were grown in poly-D-lysine coated culture plates or in flasks for individual cell type isolation. Pre-oligodendrocytes isolated from mixed glial cultures were maintained in growth medium, i.e., the serum-free Basal Defined Medium (BDM) (Dulbecco's modified Eagle's medium, 0.1% bovine serum albumin, 50 lg/mL human apo-transferrin, 50 lg/mL insulin, 30 nM sodium selenite, 10 nM D-biotin and 10 nM hydrocortisone) supplemented with platelet-derived growth factor (10 ng/mL) and basic fibroblast growth factor (10 ng/mL) for 5–9 days in order to promote progenitor proliferation (Chen et al., 2007; Kim et al., 2011).

Oligodendroyte cells cultures were set up in triplicate and were treated for 48 hours with BPA dissolved in a methanol, a medium vehicle, at concentrations of 10^{-3} M and 10^{-4} M due to preliminary data showing most obvious morphological differences at these concentrations (Chen et al., 2007). Controls were cells treated with vehicle alone. After washing. After washing with a phosphate-buffered saline and 4% paraformaldehyde, cells were immunostained with O4 antibody (1:500 dilution). Total number of cells was

revealed by staining all nuclei with Hoechst 33258. Five random consecutive fields were counted in each coverslip under 200 magnification with a total great than 1000 cells counted in the control conditions.

According to Figure 1, oligodendrocyte stages are identified by their expression of antigens identified by specific antibodies. After washing with a saline solution and fixation with a 4% paraformaldehyde for 10 minutes, cells were stained with A2B5 and Ki67 antibodies to detect oligodendrocyte precursor and proliferating cells, respectively. O4 and PLP (proteolipid protein) antibodies identify late progenitor and mature oligodendrocytes, respectively. The slides were blocked with Tris-buffered saline (TBS)-T (50 mM Tris–HCl, pH 7.4/150 mM NaCl/0.1% Triton X-100) or TBS (for O4 immunostaining) containing 5% goat serum. The coverslips were then incubated with antibody O4 (1: 500) or antibody to PLP (1: 1000) overnight at 4°C. After washes, secondary antibody conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (1: 1000 dilution, Molecular Probes, Eugene, OR, USA) was incubated with the coverslips for 1 hour at 25°C. Following more washes, nuclei were stained with Hoechst 33258 at a final concentration of 2 μg/mL for 1 min. The coverslips were then washed two to three times and mounted onto glass slides with FluoroMount and kept in the dark at 4°C. Cell images were captured with a fluorescence microscope (Olympus IX71) equipped with an Olympus DP70 digital camera (Leeds Instruments, Irving, TX, USA).

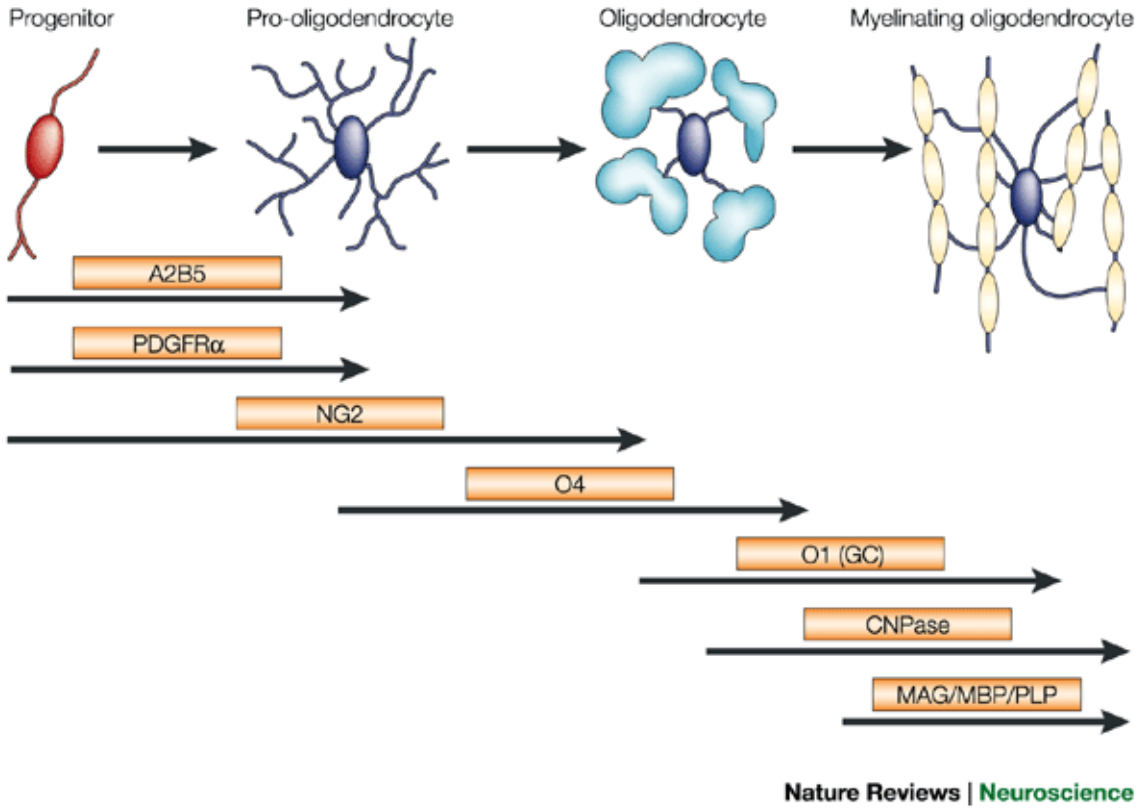

Figure 1: Defining glial cells during CNS development. The four stages of oligodendroglial lineage is differentiated by either A2B5, PDGFRα, NG2, O4, O1, CNPase, and PLP.

Cell counting software, Image J, was used to quantify samples by counting co-localized nuclei and cell bodies

CHAPTER III

RESULTS

Oligodendrocyte precursor cell cultures were assessed for maturation, proliferation, and apoptosis due to BPA exposure. To accomplish this, OPC were grown BDM media and divided up into three different treatment groups according to the molar concentration of BPA given: 10^{-3} M, 10^{-4} M, or a control group which was untreated. After 4% paraformaldehyde fixation, each experimental subgroup was subjected to immunohistochemistry to identify cells in the different stages of oligodendrocyte lineage, assess cell number, and note cell death through apoptosis.

To assess the cytotoxic effect of BPA, OPC were stained with DAPI, a DNA fluorescent stain that identifies cell nucleus with a blue color (Kapuscinski, 1995), and TUNEL staining, a red fluorescent probe that binds to apoptotic nuclei (Grasl-Kraupp et al., 2005. After imaging, images were analyzed for the percentage of TUNEL-positive nuclei present. Cell positive for TUNEL had a colocalized blue-red coloration whereas cells negative for apoptosis just appeared blue. Figure 2 is a representative image of OPC treated at a concentration of 10^{-3} M with BPA. For statistical analysis, all images were counted for the total number of TUNEL-positive cells as a percentage of the total number of cells visible in the image. Table 1 shows the averages across the three different treatment groups. However for a better representation of the group differences, Figure 3 offers a comparison.

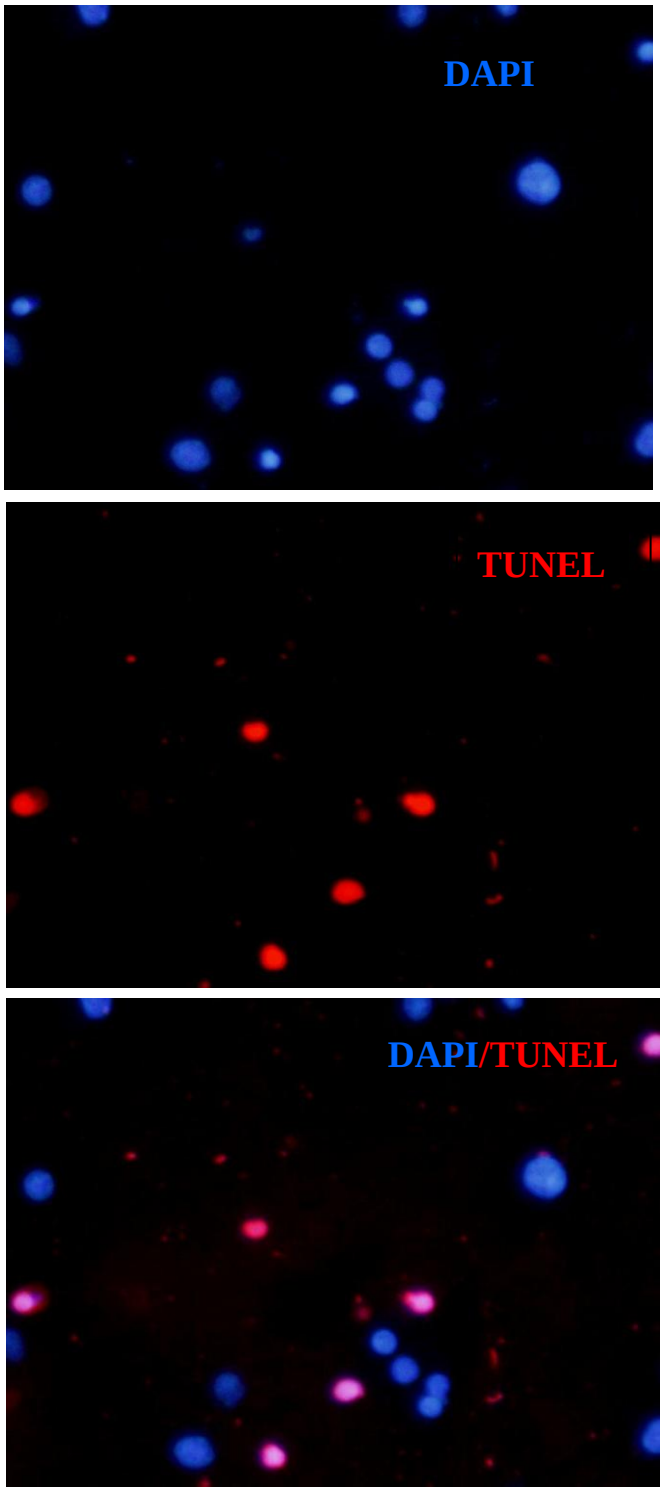

Figure 2: TUNEL immunohistochemistry for cell apoptosis. OPC treated with 10^{-3} M BPA for 48 hours. A) DAPI stain B) TUNEL stain C) DAPI and TUNEL. Representative image showing 33.3% of OPC positive for TUNEL post-treatment.

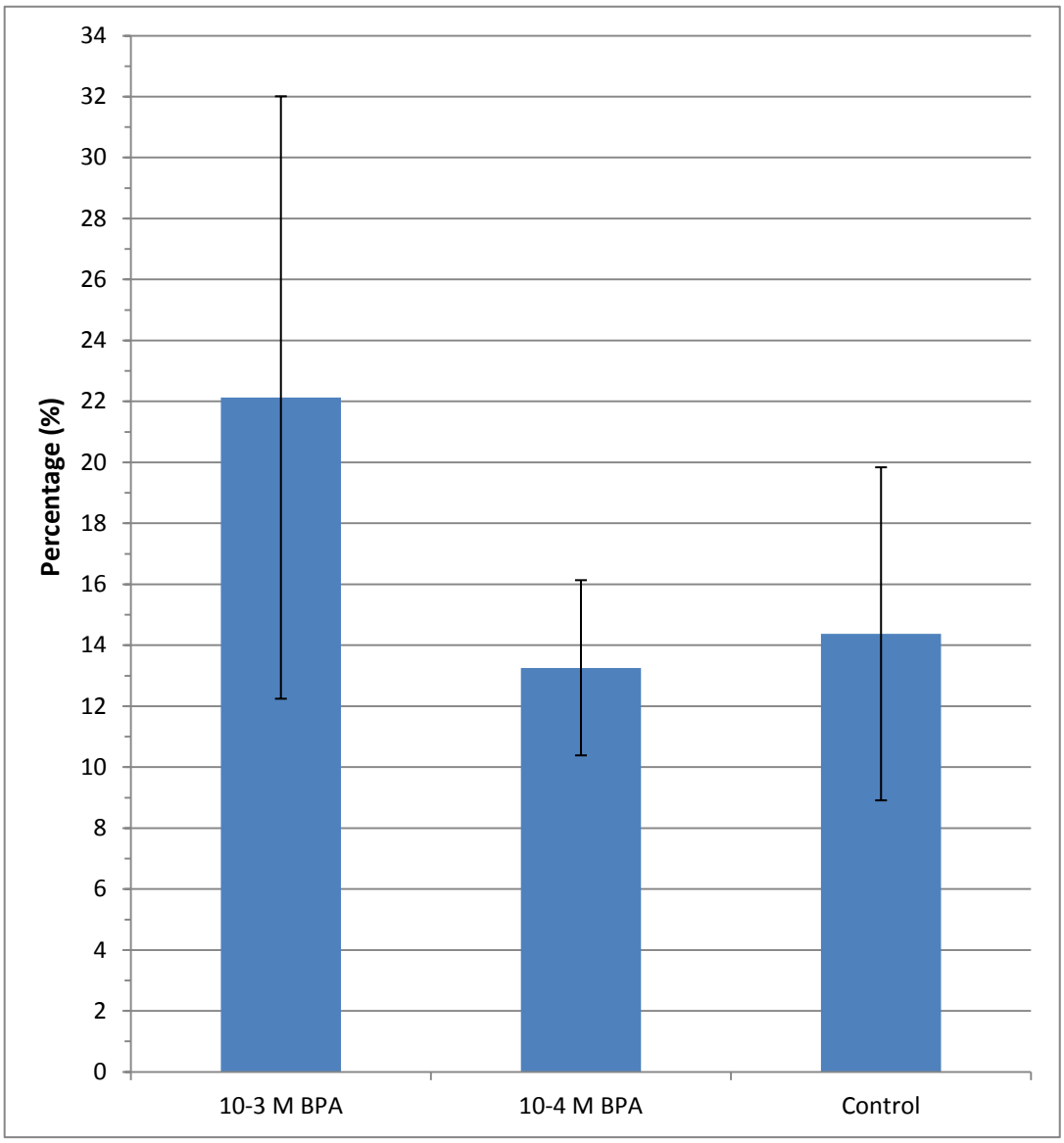

Figure 3: Comparison of Percentages of TUNEL positive cells. Groups are divided by the molar concentration of BPA administration

Table 1: Percentages of TUNEL positive cells in treatment samples. Sample groups are divided by the concentration of BPA administered. Column 1 is the average percentage of TUNEL positive in each group. Column 2 is the standard deviation of the sample percentages.

	Average	Standard Deviation
10^{-3} M BPA	22.12	9.88
10^{-4} M BPA	13.26	2.87
Control	14.37	5.46

As the trend shows, more cells are positive for TUNEL at 10^{-3} M than at 10^{-4} M and untreated cells. Samples treated with 10^{-3} M BPA had on average 22.12 ± 9.88 percent TUNEL expression. Samples treated with 10^{-4} M BPA had on average 13.26 ± 2.87 percent TUNEL expression, and samples left untreated with BPA have 14.37 ± 5.46 percent TUNEL expression. As seen in Figure 2, artifact and nuclei were objectively stained with TUNEL. These could be remnants of OPC that had lysed and left their fragments in the culture or they can be inconsequential contaminants from the medium or staining agent. Aside from the artifact, TUNEL samples were straightforward in the co-localization and counting.

To assess maturation, OPC were stained for the presence of 04, an antigen present on the pre-oligodendroglial stage of oligodendrocytes, and PLP, a protein expressed by myelinating oligodendrocytes, and again with DAPI. Although the presence of O4

proceeds that of PLP in the lineage, O4 expression continues even as the oligodendrocyte is fully mature and functional. Immunostaining of O4 appears as a red cytoplasmic stain. PLP staining is identified by a green cytoplasmic stain that when present with O4-positive cells co-localizes to produce a yellow color. DAPI verifies the presence of a nucleated cell. Figure 4 is a representative image of the staining of OPC treated for 48 hours with 10^{-3} M BPA. Each of the images stained were assessed to find the percentage of cells that fell into one of three groups: O4-positive and PLP-positive while staining with DAPI, O4-positive and DAPI-positive, or DAPI-positive. The percentages were averaged across each BPA treatment group. The results are tabulated in Table 2. For a better view of the expression of PLP or O4 across the BPA treatment groups, Figure 5 compares the percentage of cells that expressed a certain phenotype depending on the concentration of BPA administered for 48 hours.

Table 2: Average Percentages for O4-positive and PLP-positive cells.

		PLP+O4+DAPI	**O4+DAPI**	**DAPI**
10^{-3} M BPA	Average	44.46	52.24	3.31
	Standard Deviation	18.13	17.23	3.99
10^{-4} M BPA	Average	41.43	51.45	7.12
	Standard Deviation	9.36	6.55	4.90
Control	Average	50.04	41.97	7.99
	Standard Deviation	15.00	12.07	5.23

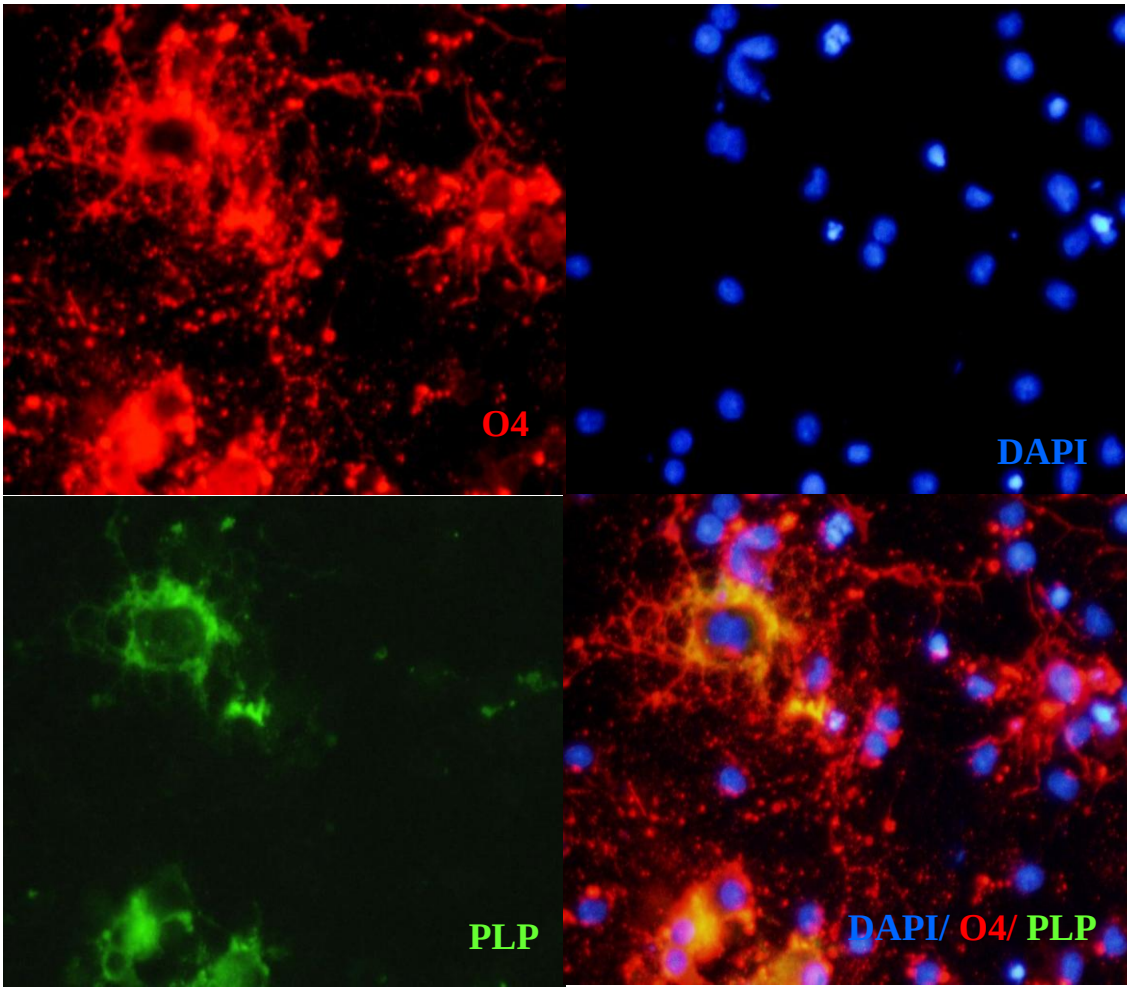

Figure 4: O4 and PLP immunohistochemistry for cell maturation. OPC treated for 48 hours with 10^{-3} M BPA. A) O4 stain B) DAPI stain C) PLP stain D) merged image with DAPI, O4, and PLP. Representative image shows that 33.3% of OPC express all three antigens, 63.3% express only O4 and DAPI, and 3.33% express

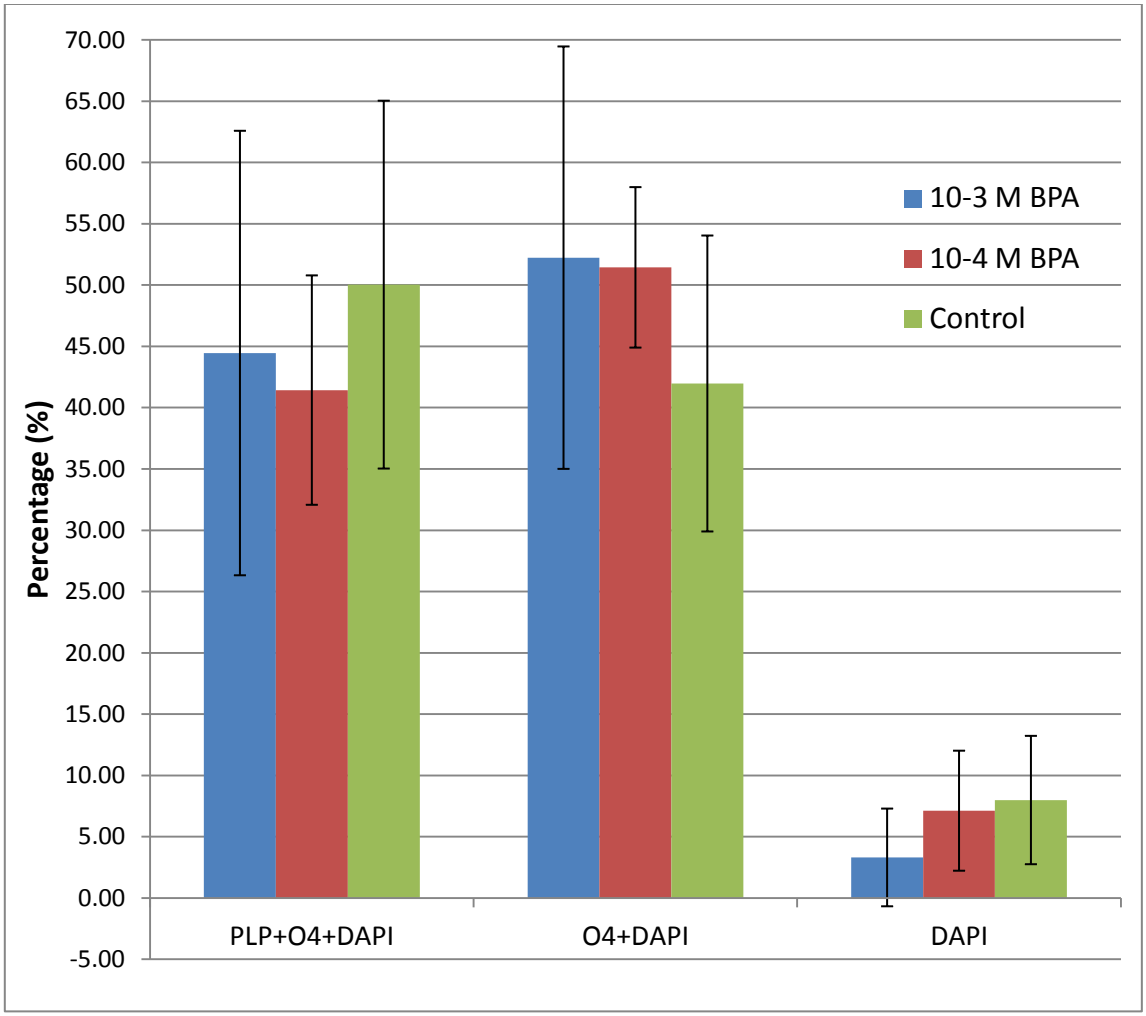

Figure 5: Comparison of Average Percentages of PLP and O4 expression. Groups are organized based on which concentration of BPA was administered as well as the expression of a combination of antigens.

Expression of PLP, O4, and DAPI is the highest in the control group, second highest in the cells treated at 10^{-3} M, and least of all by the OPC treated at 10^{-4} M. O4 and DAPI expression is the highest at a concentration of 10^{-3} M, then 10^{-4} M, and least expressed on the control group. There are more cells that only express DAPI in the control group than in the 10^{-3} M treatment group and 10^{-4} M treatment group.

To assess proliferative effects of BPA on OPC, cells were immunostained for the presence of A2B5, Ki67, and DAPI. A2B5 is an antigen present on oligodendrocyte precursor cells and Ki67 is present on all cells that are participating in the proliferative cell cycle. The only cells in the oligodendrocytes lineage that actively divide or have recently divided are the oligodendrocytes precursor cells. A2B5 is a red cytoplasmic immunostain. Ki67 is a green nuclear stain that varies in its intensity of expression depending on the stage in which the cell is currently present (Brown and Gatter, 1990). Figure 6 offers a representative sample of OPC treated with 10^{-3} M BPA and assessed for immaturity and proliferation. The cells are group based on whether they fall into one of two groups: Ki67-positive and A2B5-positive while expressing DAPI or A2B5-positive and DAPI-positive. Table 3 summarizes the average findings for each of the treatment groups. As evidenced by the data, nearly 100% of each of the treatment groups expressed Ki67 and A2B5. There were not any samples that had cells that solely expressed DAPI without A2B5.

Table 3: Average Percentages for Ki67-positive, A2B5-positive for cell proliferation

		DAPI+A2B5+ Ki67	A2B5 + DAPI
10^{-3} M BPA	Average	99.8	0.2
	Standard Deviation	0.5	0.5
10^{-4} M BPA	Average	99.8	0.2
	Standard Deviation	0.4	0.4
Control	Average	99.5	0.5
	Standard Deviation	0.9	0.9

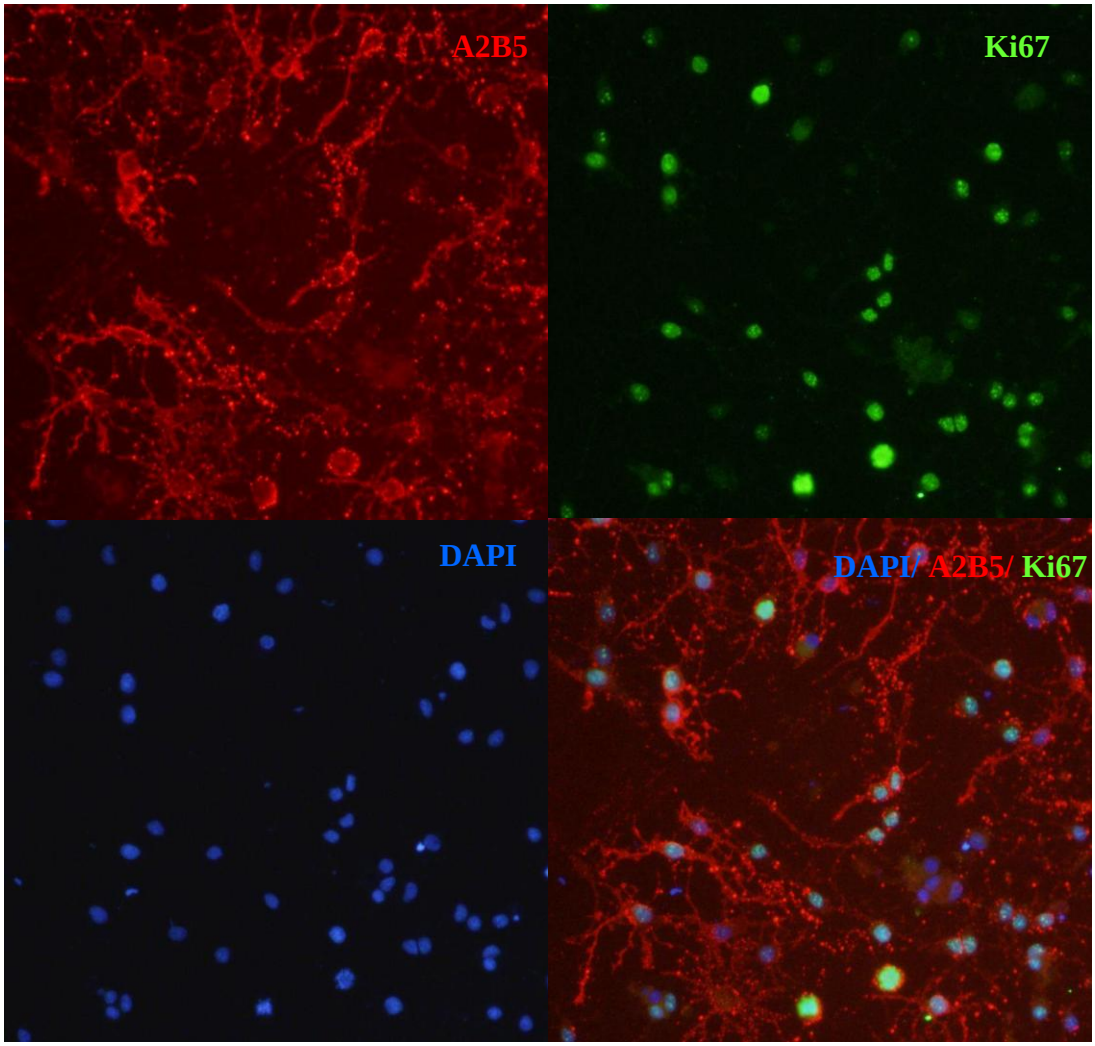

Figure 6: Immunohistochemical analysis for cell proliferation and immaturity with A2B5, Ki67, and DAPI. OPC were treated for 48 hours with 10-3 M BPA.

The greatest problem posed to the acquisition of the results came from the images themselves. Many of the images captured by immunofluorescence appeared blurry. Also the immunostains for specific antigens often stained the background, giving the appearance that everything in the sample included the stain. To reconcile this issue, all cells that expressed even a faint level of immunofluorescence that indicated the presence of an antigen were counted as positive for the antigen. This was done because physiologically the faint expression of an antigen is indicative of the cell stage and should not be ignored. Furthermore many of the images had greater than 150 cells which is not an ideal range of values to attempt to get statistically significant differences in counts because the difference of ten cells holds greater value at lower counts than at higher counts where the difference is dilute.

CHAPTER IV

CONCLUSIONS

One of the aims of this project was to assess whether or not BPA had cytotoxic effects on OPC. If BPA caused cell death in oligodendrocytes, then that may provide an understanding of the increased incidence of multiple sclerosis and other neurological disorders associated with malfunctioning of myelinated neuronal pathways. The data showed that at high levels of BPA around 10^{-3} M, cells showed a 22% expression of TUNEL, therefore showing that around 22% of cells exposed to BPA underwent apoptosis. This value is higher than those found at 10^{-4} M or untreated samples which each had about 13% and 14% apoptosis, respectively. Therefore it can be assumed that high levels of BPA may induce apoptosis in oligodendrocyte precursor cells.

However, the nature of the TUNEL stain itself poses a challenge to data gathered. TUNEL binds to spliced ends of DNA that become exposed after DNA terminal deoxyribonucleotidyl transferase has cleaved DNA. However TUNEL cannot differentiate between apoptosis, necropoptosis, or autolysis (Grasl-Kraupp et al., 2005). Therefore we are unable to definitively assess the mode of the cell death observed. However the fact that we observed dose response of more cell death in the higher concentrations of BPA suggests that BPA is having a toxic effect on oligodendrocytes.

BPA has been previously proven to cause the early maturation of astrocyte progenitor cells *in vitro* (Yamaguchi et al., 2006). If the same trend of BPA applies to oligodendrocyte precursor cells, then the early maturation of this endocrine disrupting compound may cause premature myelination of incomplete pathways in neonates/ infants. To study this effect, OPC cultures treated at different concentrations were immunostained with antibodies to proteolipid protein (PLP) and O4. O4 is present on the proligodendrocytes that have exited the cell cycle, ceased cell proliferation and migration, grown several processes, and lacks expression of A2B5 on the cytoplasm. PLP is only present on the terminal stage of the oligodendrocytes lineage on cells that are actively myelinating axons. The presence of one or both of these antigens through the immunohistochemical assay is indicative of early maturation.

According to the data collected the percentage of immature oligodendrocytes that lack both PLP and O4 expression in all three treatment groups, on average, falls under 10% of the total cell population. Cultures treated with 10^{-3} M BPA have the least amount of immature oligodendrocytes at an average percent of 3.31%. Cells that are left untreated and that have a lower BPA administration average around 7.99% and 7.12% respectively. This result was predicted by previous publications. Interestingly, the control samples had the highest average percentage of mature oligodendrocytes that expressed PLP, O4, and DAPI around 50%. It was expected that cells that were treated with BPA should express the higher percentage of fully mature oligodendrocytes. This anomaly in the results can arise from a number of sources, but probably came from

human error in data interpretation. Cells treated with 10^{-3} M and 10^{-4} M of BPA had similar average percentage of O4-positive and DAPI-positive cells at 52.24% and 51.45%, respectively. However, cells treated with 10^{-3} M BPA had the greatest range and the highest average percentages of the experimental samples. This trend follows what was predicted, showing that higher concentrations of BPA cause OPC to mature into proligodendrocytes.

BPA imitates estrogen on the thyroid gland and decreases the output of Thyroid Hormone. Thyroid Hormone plays a key role in the maturation of oligodendrocytes so the inhibition of thyroid hormone will inhibit the maturation of oligodendrocytes by proxy. The final aim to assess the proliferation of OPC after exposure to BPA highlights the fact that immature progenitor oligodendrocytes that express A2B5 will be the cells undergoing the most proliferation and expressing Ki67. Ki67 is present on all stages of the proliferative cell cycle at varying degrees depending on the stage. However, Ki67 is not present on cells that have exited the cell cycle, are arrested at G0-phase, and have stopped proliferation (Brown and Gatter, 1990).

According to the data acquired through the final aim, essentially 99% of the cells in samples treated with 10^{-3} M BPA, 10^{-4} M BPA, and untreated cells displayed A2B5 and Ki67. Therefore, regardless of treatment of BPA, a majority of OPC remained immature. It would have been expected to see that only those cultures treated with BPA stayed in their proliferative, immature cycle, but even the untreated cells expressed

A2B5 and Ki67. This can be attributed to the fact that oligodendrocytes *in vitro* often stay immature for up to 3 days, at which point they stop expressing A2B5 and begin expression of O4. As previously mentioned, the difficultly in deciding whether to count the faded stain as artifact or significant probably contributed to the high immature population in the untreated control samples.

The data gained from this project has certainly shown the influence of BPA exposure on oligodendroglial lineage progression. BPA has caused OPC to undergo apoptosis or related cell death. It has also caused OPC to mature at a faster rate and decrease the concentration of immature cells *in vitro*. Furthermore it has caused the same OPC to remain in a period of cell immaturity and proliferation. In conclusion, low doses of BPA have a detrimental effect on oligodendrocytes viability and development, which may contribute to the pathogenesis of multiple sclerosis and explain, in part, the increased incidence of disease.

Future directions

The data gathered has opened up new information about the physiological changes due to BPA. But there is a need to delve into the root of the matter and explore the changes BPA poses on the genetic level. In a future experiment, it will be useful to extract RNA from in vitro cultures of OPC treated with BPA as well as in vivo cultures of mouse OPC fed BPA. The extracted RNA can then be analyzed for epigenetic changes such as methylation, acetylation, and other chromatin modifications as induced by BPA exposure. This information will provide thorough information about any additional effects of BPA on OPCs.

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